Patent Application
Docket No. SPO.129

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Philip Dubois
Art Unit	:	1781
Applicants	:	Hisae Kume *et al.*
Serial No.	:	10/593,550
Filed	:	September 19, 2006
Conf. No.	:	4478
For	:	Antibacterial Compositions

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF HISAE KUME UNDER 37 CFR §1.132

Sir:

I, Hisae Kume, hereby declare:

THAT, I am a Research Scientist;

THAT, my qualifications are set forth in more detail in Exhibit 1 (attached hereto);

THAT, I am an inventor on the above-referenced application;

THAT, I, have reviewed the specification, the pending claims, the reference cited, and the Office Action mailed September 3, 2010;

And being thus duly qualified, do further declare as follows:

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And being thus duly qualified, do further declare as follows:

Our invention provides a novel antibacterial composition. The antibacterial composition is prepared using a fermented dairy product, and has a pH of 4.6 or less. The composition comprises carbohydrates, proteins, and fats, wherein the energy ratio of carbohydrates, proteins, and fats is 50% to 70%, 4% to 25%, and 20% to 30%, respectively.

In this Declaration, I report results of a comparative experiment, which compares the antibacterial activity of our composition (the acidic liquid diet of Table 1) to various control compositions of Table 2 (*e.g.*, neutral liquid diet, liquid fermented milk preparation, jellylike total nourishing diet, Yogurt 1, Yogurt 2, solid fermented milk, and liquid fermented milk). These control compositions contain carbohydrates, proteins, and fats, which are present within the ranges of concentrations described in Izvekova *et al.*

The results, as shown in Figure 1, demonstrate that our composition possesses unexpectedly superior antibacterial effects, when compared to these control compositions.

Materials and Methods

Staphylococcus aureus IID 1677 (MRSA) were cultured in regular agar medium (Eiken Chemical). MRSA suspension was prepared by suspending bacterial cells in sterilized physiological saline solution. The number of bacterial cells per 1 mL of suspension was adjusted to approximately 4.4×10^7.

The antibacterial composition of our invention (the acidic liquid diet) was prepared by mixing the ingredients of Table 1. The fermented dairy product - quark was prepared as follows. First, skimmed milk was inoculated with 1% of lactobacilli starter (a combined starter of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*), and fermented at 35°C for 16 hours, thereby producing fermented curd. The curd was then placed in a quark separator, and quark containing approximately 13% proteins, 0.3% fats, and 5% carbohydrates was obtained. Approximately 19% total solids were obtained by separating whey protein from the quark. Mixed oils and fats of Table 1 comprise fatty acids such as palmitic acid, oleic acid, linoleic acid, linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid. The percentage of fatty acids with double bonds is 25% and the ratio of n-6/n-3 fatty acids is 7.4%.

A neutral liquid diet and various acidic fermented dairy products (*e.g.*, liquid fermented milk preparation, jellylike total nourishing diet, Yogurt 1, Yogurt 2, solid fermented milk, and

liquid fermented milk of Table 2) were used as control compositions. These control compositions comprise carbohydrates, proteins, and fats, which are present within the ranges of concentrations described in Izvekova *et al.* (see Izvekova *et al.* at column 5, lines 20-26). Among these acidic fermented dairy products, the liquid fermented milk preparation was prepared by adding sterile distilled water to quark, and the mixture was sterilized at 95°C for 5 minutes. The liquid fermented milk preparation comprises quark at a concentration of 33 g/100 mL, which is the same as the quark concentration of the acidic liquid diet (Table 1).

Table 1

Ingredients	Blended Quantity (per 100 mL)
Fermented dairy product	33.4 g
Honey	8 g
Dextrin	6.1 g
Sucrose	1 g
Indigestible dextrin	0.61 g
Pectin	0.75 g
Mixed oils and fats	2.6 g
Soybean lecithin	0.13 g

Table 2

	Manufacture	kcal/100g	Protein	Fat	Carbohydrate	pH(measured value)
Neutral liquid diet	Meiji Dairies Corporation	100	4	2.8	15.5	6.68
Acidic liquid diet	Meiji Dairies Corporation	100	4	2.8	15.6	4.06
Liquid fermented milk preparation	Meiji Dairies Corporation	46	4	0	7.5	4.06
Jellylike total nourishing diet	Meiji Dairies Corporation	100	4	2.8	14.4	3.66
Yogurt 1	Bright Dairy & Food Co.	85	3	3.2	11	4.53
Yogurt 2	China Mengniu Dairy Company	85	2.9	3.1	11.3	4.17
Solid fermented milk	Meiji Dairies Corporation	62	3.4	3	5.3	4.21
Liquid fermented milk	Meiji Dairies Corporation	67	3.1	0.5	12.6	4.15

To examine the antibacterial effects of our composition (the acidic liquid diet), 1 mL of MRSA suspension was added to 100 mL of the acidic liquid diet and control compositions, respectively. The MRSA-containing compositions were cultured at 37°C. After 3, 6, and 24 hours of culturing, the number of viable bacterial cells/ml was determined as follows. First, the MRSA-containing compositions were diluted with SCDLP medium (Nihon Pharmaceutical). The diluted compositions were cultured on an agar plate at 35 ± 1°C for 2 days using pour-plate techniques. Two days later, the number of developing colonies/ml was counted. Sterilized physiological saline solution was used as control.

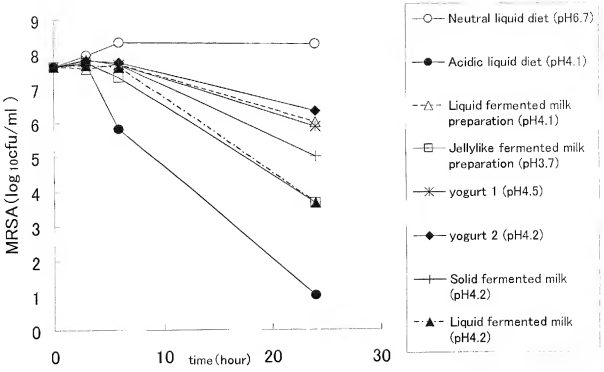
Results

The results demonstrated that the composition of our invention (the acidic liquid diet) has unexpectedly superior growth suppressive effects against Gram-positive bacteria *Staphylococcus aureus* IID 1677 (MRSA), when compared to the neutral liquid diet and acidic fermented dairy products comprised of carbohydrates, proteins and fats present within the ranges of concentrations described in Izvekova *et al.*

As shown in Figure 1, the acidic liquid diet of our invention reduced *Staphylococcus aureus* IID 1677 cell count from $4.4x10^7$ cells/ml to "less than 10 cells/ml" which means in the art that cells were undetectable, after 24-hour incubation. (Note: only for the jellylike total nourishing diet (pH3.7), cfu/g (gram) was used instead of cfu/ml because the viscosity was very high.)

In comparison, various acidic fermented dairy products had little or almost no growth suppressive effects against *Staphylococcus aureus* IID 1677. For example, after 24-hour incubation with the products other than our composition, *Staphylococcus aureus* IID 1677 cell count remained as high as **about 10^4 to 10^8 cells/ml**. Since *Staphylococcus aureus* IID 1677 cells were **undetectable** after the incubation with our composition, it is clearly demonstrated that our composition possesses unexpectedly superior effects.

Figure 1


MRSA (log 10cfu/ml)
9
8
7
6
5
4
3
2
1
0
0
10
time (hour)
20
30
Neutral liquid diet (pH6.7)
Acidic liquid diet (pH4.1)
Liquid fermented milk preparation (pH4.1)
Jellylike fermented milk preparation (pH3.7)
yogurt 1 (pH4.5)
yogurt 2 (pH4.2)
Solid fermented milk (pH4.2)
Liquid fermented milk (pH4.2)

I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the specification or any patent issuing thereon.

Dated: November 1, 2010

Hisae Kume

Attachment: Exhibit 1 (Statement of Qualifications)

Support of the Declaration of Hisae Kume, Ph.D. Under 37 C.F.R. § 1.132

Resume of Hisae Kume, Ph.D.

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Research assistant of Health Science Institute
January 1989 to September 1991: University of Colorado USA
Research assistant of Dr. Sueoka's Lab. (MCDB)

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* : The paper(12) are related to the patent.

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Research Article

Antibacterial Activity of Some Lactic Acid Bacteria Isolated from an Algerian Dairy Product

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Recommended by Benny C. Zee

In the present study, the antibacterial effect of 20 lactic acid bacteria isolates from a traditional cheese was investigated. 6 isolates showed antibacterial effect against Gram positive bacteria. *Streptococcus thermophilus* T2 strain showed the wide inhibitory spectrum against the Gram positive bacteria. Growth and bacteriocin production profiles showed that the maximal bacteriocin production, by *S. thermophilus* T2 cells, was measured by the end of the late-log phase (90 AU ml^{-1}) with a bacteriocine production rate of 9.3 (AU ml^{-1}) h^{-1}. In addition, our findings showed that the bacteriocin, produced by *S. thermophilus* T2, was stable over a wide pH range (4–8); this indicates that such bacteriocin may be useful in acidic as well as nonacidic food. This preliminarily work shows the potential application of autochthonous lactic acid bacteria to improve safety of traditional fermented food.

1. Introduction

Lactic Acid Bacteria (LAB) isolated from dairy products have received increased attention as a potential food preservative due to their antagonistic activity against many food born pathogen such as *Listeria monocytogenes* [1]. LAB are widely distributed in the nature, they are typically involved in a large number of the spontaneous food fermentation, and they have been extensively studied [2]. Some members of LAB produce bacteriocins and bacteriocins-like substances which may inhibit growth of spoilage and pathogenic microorganisms [3]. Bacteriocins from LAB are bioactive peptides or proteins with antimicrobial activity toward Gram positive bacteria, including closely related strains and/or spoilage and pathogenic bacteria [4]. Bacteriocins are ribosomaly synthesized and extracellulary released bioactive peptides or peptide complexes which have bactericidal or bacteriostatic effect [5]. Use of either the bacteriocins or the bacteriocin-producing LAB like starter cultures for food preservation has received a special attention [6]. Moreover, bacteriocins are innocuous due to proteolytic degradation in the gastrointestinal tract [7, 8]. *S. thermophilus* is a lactic acid bacterium of major importance in food industry like the manufacture of yoghourt [9]. Some of *S. thermophilus* strains produce a bacteriocin named thermophilin which is active against several LAB and food spoilage bacteria such as *Clostridium sporogenes*. In view of its technological and biochemical properties the above bacteriocin can be considered as a potential biopreservative [10]. Some of other LAB like *Enterococcus*, *Lactococcus*, and *Pediococcus* are also widely used as natural preservatives, due to the potential production of metabolites with antimicrobial activity such as organic acids, hydrogen peroxide, antimicrobial enzymes and bacteriocins [11].

The aim of the present study is to assess antimicrobial activity of some lactic acid bacteria strains isolated from traditional fermented dairy products prepared from raw milk, Raib, which is obtained after spontaneous curdling of raw milk within 24 to 36 hours at ambient temperature. In addition, preliminary investigations on a bacteriocin produced by *S. thermophilus* strain isolated in this work will be presented.

2. Materials and Methods

2.1. Isolation of Lactic Acid Bacteria. The bacterial strains used in this study were isolated from fermented traditional milk, Raib, manufactured without starter cultures. Samples were collected all over Chlef regions and obtained with collaboration of Bioressources research laboratory. LAB were isolated from Raib, by homogenizing 10 g samples of cheese in 90 mL saline solution and then plating suitable serial dilutions onto different media: BHI, MRS, and M-17 (Biokar Diagnostics, Beauvais, France). The plates were incubated aerobically at 30°C for 48 hours, and then several colonies were picked at random for identification. Cell morphology and Gram-staining reaction were examined by light microscopy, and the catalase activity was carried out. Phenotypic identification was based upon physiological and biochemical characteristics; sugar fermentation profile, in the API-20 Strep CH and API-50 CH fermentation, was carried out according to the manufacturer's instructions (bioMe'rieux, Marcy l'Etoile, France).

2.2. Detection of Antibacterial Activity. For detection of antagonistic activity, an agar spot test was used. The agar spot test was a modification of that described by Tomé et al. [12]. Overnight cultures, on MRS medium, of the strains to be tested for production of antimicrobial compound were centrifuged (10 minutes at 15000 g, 4°C). Cell-free supernatants were filtered across cellulose acetate filter (0.2 μm) to remove residual cells.

An overnight culture (37°C) of the target strain was diluted in sterile Mueller-Hinton Medium, and 2 mL of $ca \cdot 10^6$ CFU mL^{-1} were spread on solid Mueller-Hinton medium. After 5 minutes of contact, the excess was removed and the Petri dishes were dried for 10 minutes. Samples (10 μL) of filtered cell-free supernatants were spotted on the agar plate. The target strains used in this study are *Bacillus cereus* CIP 6624, *Bacillus subtilis* ATCC 6633, *Escherichia coli* CIP 35218, *Enterococcus faecalis* CIP 29212, *Listeria innocua* ATCC 51742, *Salmonella typhimurium* CIP 5858, *Staphylococcus aureus* CIP 29213, *and Staphylococcus epidermitidis* ATCC 14990.

2.3. Sensitivity of Bacteriocin to Enzymes, pH and Heat Treatment. The biochemical nature of the antibacterial agent was studied on both chloroform extract and cell-free supernatant; all the samples were incubated for 1 hour at 37°C before the antilisterial essay. The pH of cell-free supernatants was adjusted to 6.5 with NaOH (1 N) and then treated with catalase (Sigma; 500 IU mL^{-1}). The cell-free supernatant was also submitted to heat treatment (60–95°C) and to several pH (4–8). The chloroform extract was treated with α-amylase (Sigma; 1 mg mL^{-1} 100 mM phosphate buffer, pH 6.9), α-chymotrypsin (Sigma, 1 mg mL^{-1}, 0.05 M Tris–HCl buffer (pH 8.0)–0.01 M $CaCl_2$), Pronase E (Sigma; 1 mg mL^{-1} in 100 mM Tris–HCl buffer, pH13), Proteinase K (Sigma; 1 mg mL^{-1} in 100 mM Tris–HCl buffer, pH 7.5), and Trypsin (Sigma, 1 mg mL^{-1} 50 mM Tris–HCl buffer pH 8.0). Prior to being assayed for bacteriocin activity, preparations containing pronase E were adjusted to pH 6.0. Neutralized cell-free supernatant neutralized cell-free supernatant treated with catalase, heat-treated supernatant, and chloroform extract were spotted against *L. innocua*. The enzymes were heat inactivated for 3 minutes at 100°C. For each test, untreated bacteriocin plus buffer, bacteriocin plus buffer treated 5 minutes at 100°C, buffer alone and enzymes solutions served as controls [13, 14].

2.4. Growth Kinetic and Bacteriocin Production. Growth experiments were performed in ErlenMeyer flask of 500 mL containing 250 mL of MRS broth (pH 6.5) at 37°C without shaking. An overnight pre-culture of *S. thermophilus* was used for the inoculation of the MRS broth at initial cell density of $ca \cdot 10^3$ CFU mL^{-1}. At different time intervals, samples were removed from the culture and used for optical density measurement (660 nm), viable and cultivable count (CFU mL^{-1}), extracellular pH measurements, and bacteriocin production. The antibacterial concentration of each sample was conducted with the critical method of dilutions [15]. The bacteriocin concentration Arbitrary Unit mL^{-1} (AU mL^{-1}) was calculated as the inverse of the strongest dilution which induces the inhibition of *L. innocua*. All experiments were repeated at least three times. The experiments were repeated three times, and results are expressed as mean ± standard error to the mean.

2.5. Bacteriocin Extraction. The extraction was realized from cell-free culture supernatant of *S. thermophilus* obtained after centrifugation of overnight culture (20 minutes at 15000 g at 4°C). The extraction was performed according to Buriaack and Yousef [16]. The culture supernatant (100 mL) was stirred vigorously for 20 minutes with chloroform (v/v) and transfer in separation funnel, the interface layer between the aqueous and organic phases, which contain bacteriocin, was harvested, and the residual chloroform was eliminated by speed vacuum (50 hours, Unique, Martinsried, Germany). Then bacteriocin activity was measured in the interface layer, aqueous and organic phases.

2.6. Plasmid Extraction. The plasmid extraction was performed from a cell pellet of an overnight culture of *S. thermophilus* (250 mL) using Miniprep Spin kit together with the corresponding buffers purchased from QIAGEN (Hilden, Germany). *S. thermophilus* plasmidic DNA analysis was performed by electrophoresis (1 hour, 100 V) using a 0.7% agarose gel dissolved in Tris 45 mM; Borate 45 mM; EDTA 1 mM; pH 8. The electrophoresis gels were analyzed under UV using Molecular Imager Gel Doc System (Bio-Rad, Hercules, USA).

2.7. HPLC Purification of Supernatant Chloroform Extract. The conditions for bacteriocin isolation were realized, through analytical RP-HPLC, on the chloroform extract. The liquid chromatographic system consisted of a Waters 600 E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device, and a Waters 996 photodiode array detector. Spectral and chromatographic data were

Table 1: Lactic acid bacteria isolated from traditional dairy product (Raib).

Strain	Source	Growth medium
Lactococcus lactis S1	Raib	MRS
Lactococcus lactis S2	Raib	MRS
Lactococcus lactis S3	Raib	MRS
Lactococcus lactis S4	Raib	MRS
Lactococcus lactis S5	Raib	MRS
Lactococcus lactis S6	Raib	MRS
Lactococcus lactis S7	Raib	MRS
Lactococcus lactis S8	Raib	MRS
Lactococcus lactis S9	Raib	MRS
Lactococcus lactis S10	Raib	MRS
Lactococcus lactis S11	Raib	MRS
Lactococcus lactis S12	Raib	MRS
Lactococcus lactis S13	Raib	MRS
S. thermophilus T1	Raib	MRS
S. thermophilus T2	Raib	MRS
S. cremoris R1	Raib	MRS
S. cremoris R2	Raib	MRS
S. cremoris R3	Raib	MRS
Lactococcus diacetylactis V1	Raib	MRS
Lactococcus diacetylactis V2	Raib	MRS

stored on a NEC image 466 computer. Millennium software was used to plot, acquire, and analyze chromatographic data.

All of the chromatographic processes were performed on an Uptisphere C_{18} column (150 mm×4.6 mm, UP5ODB615QS, Interchim, Montluçon, France). The mobile phase was water/trifluoroacetic acid (1000 : 1, v/v) as eluent A and acetonitrile/trifluoroacetic acid (1000 : 1, v/v) as eluent B. The flow rate was 1 mL/min^{-1}. Samples were filtered through 0.22 μm filters and then injected. The gradient applied was 0–50% (v/v) B over 100 minutes then 50%–100% (v/v) B over 5 minutes and 15 minutes at 100% (v/v) B. Online UV absorbance scans were performed between 200 and 300 nm at a rate of one spectrum per second with a resolution of 1.2 nm. Chromatographic analyses were completed with Millennium software [17].

3. Results

3.1. Antimicrobial Activity. Twenty LAB strains, isolated from Algerian dairy milk (Table 1), were screened for their antagonistic activity against *Listeria innocua*, *Enterococcus faecalis*, *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermitidis*, *Escherichia coli*, *and Salmonella typhimurium*. The results of Table 2 show that six isolates were active against one or more tested strains. However, *S. thermophilus* T2 strain showed a wide inhibitory spectrum against all the Gram positive target bacteria used in this study except against *Staphylococcus aureus* (Table 2). In addition, *S. thermophilus* T2 did not show any inhibitory activity against Gram negative bacteria used in this study: *Escherichia coli*and*Salmonella typhimurium*.

3.2. Nature of the Inhibitory Agent. Our results showed that the free-cell supernatant remained active, against sensitive target strains, even when the pH was adjusted to pH 7. However, when the cell-free supernatant and the chloroform extract were exposed to the proteolytic enzymes (Table 3) no inhibitory activity was observed against *Listeria innocua* by contrast to the control tests which showed an inhibitory activity against the target strain (Table 3). In addition, when the cell-free supernatant and the chloroform extract were exposed to the action of α-amylase and catalase similar inhibitory activity was measured when compared with the control test against *L. innocua*. These results suggest that the biochemical nature of the molecule produced by *S. thermophilus* is peptidic. Moreover, the antimicrobial activity appeared to be heat resistant. Thus, the inhibitory activity of the chloroform extract was still measured after a heat treatment of 30 minutes at 90°C. Our results showed also that in a range of pH 4–8 similar antibacterial activities of the chloroform extract were obtained against *L. innocua*.

3.3. Extraction of the Bacteriocin Produced by S. thermophilus. The extraction of the bacteriocin produced by *S. thermophilus* T2 strain from culture supernatant was realized with chloroform, a water-immiscible solvent. The method used concentrates the bacteriocin at the interface between chloroform and the aqueous culture of the producing bacterium. We demonstrated that no bacteriocin activity was detected in the solvent phase (data not shown). In addition, the precipitate at the interface between the chloroform and culture supernatant fluid contained most of the bacteriocin activity in the mixture. The precipitate at the interface was harvested, and the residual chloroform was eliminated by speed vacuum. After HPLC reversed-phase chromatography, bacteriocin activity was associated with two peaks eluting at 17 minutes and 110 minutes (Figure 3). These results showed that the antibacterial activity of *S. thermophilus* T2 could be associated with two molecules which present different hydrophobicity.

3.4. Growth Kinetics and Bacteriocin Biosynthesis. Growth and bacteriocin production of *S. thermophilus* was studied in MRS broth at 37°C at pH 6.5. Under these conditions bacteriocin activity was detected at 4 hours of incubation at the beginning of the exponential phase, at a cell concentration of $ca{\cdot}10^4$ CFU mL^{-1} (12 AU mL^{-1}). The results of Figure 1 showed that bacteriocin production increases with the increase of cell concentration to reach a maximum of 90 AU mL^{-1} with a bacteriocin production rate of 9.3 (AU mL^{-1}) h^{-1}. This concentration was reached between 12 and 14 hours of incubation at 37°C. During the stationary phase both bacteriocin concentration and the cell concentration remained at a steady state (Figure 1). Antibacterial activity decreased after 24 hours of incubation after having reached maximum levels after 14 hours of incubation (data not shown).

Table 2: Antibacterial spectrum of the cell-free supernatant of the six lactic acid bacteria isolated from the traditional dairy product (Raib).

Strain	Strains inhibited
Lactococcus lactis S1	*Listeria innocua, Enterococcus faecalis*
Lactococcus lactis S2	*Listeria innocua*
Lactococcus lactis S7	*Listeria innocua, Enterococcus faecalis Bacillus cereus*
Lactococcus lactis S9	*Listeria innocua, Bacillus cereus*
S. thermophilus T2	*Bacillus cereus, Bacillus subtilis, Listeria innocua, Enterococcus faecalis, and Staphylococcus epidermitidis*
S. cremoris R3	*Enterococcus faecalis, Bacillus cereus*
Lactococcus diacetylactis V1	*Enterococcus faecalis*

Table 3: Effect of different treatments on cell-free supernatant and chloroform extract of *S. thermophilus* T2. Relative activity was measured by an agar diffusion test against *Listeria innocua*. (−): no inhibition; (+): slight inhibition; (++): moderate inhibition; (+++): strong inhibition.

Treatments	Relative activity
Enzymatic treatments	
Proteinase K	−
Pronase E	−
α-chymotrypsin	−
Trypsin	−
α-amylase	++
Catalase	++
Control	+++
pH treatments	
4	+++
5	+++
6	+++
7	+++
8	+++
Control	+++
Heat treatments	
60°C	+++
70°C	+++
80°C	++
90°C	++
95°C	+
Control	+++

3.5. Plasmid Content. The genes encoding for bacteriocin are either chromosomic or plasmidic [18, 19]. The aim for this preliminary investigation is to assess the presence of plasmid in *S. thermophilus* cells. The analysis of the plasmidic DNA extraction showed that *S. thermophilus* seems to have a single plasmid as shown in Figure 2. In addition, *Dra*I fragmentation pattern of the *plasmid* resulted in three *restriction fragments* with approximately 2.2 kb, 1.5 kb, and 0.5 kb. Thus the size of the plasmid could be of at least 4.2 kb. This preliminary result is of importance since in many lactic acid bacteria bacteriocins and carbohydrate fermentation exopolysaccharide production and antiphage mechanisms are carried by the same plasmid as reported previously by Martinez-Bueno et al. [20] and Turgeon and Moineau [21].

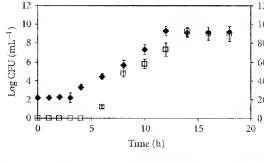

Figure 1: Growth kinetic and bacteriocin production by *S. thermophilus*. The growth was performed at an initial pH of 6.5, at 37°C without shaking. (♦) growth kinetic. (□) bacteriocin production. The experiments were repeated three times and results represent the mean ± standard error to the mean.

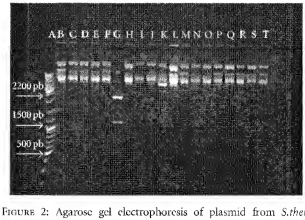

Figure 2: Agarose gel electrophoresis of plasmid from *S.thermophilus* digested by various restriction endonucleases: (A) O'Gene Ruler control (B); *Ava*III (C); *Bam*HI ; (D) *Bgl*II ; (E) *Bst*EII; (F) *Eco*RI, (G) *Dra*I; (I1) *Eco*RV; (I) *Hind*III; (J) *Nde*I; (K) *Mfe*I; (L) *Pst*I, (M) *Pvu*II; (N) *Sac*I; (O) *Sca*I; (P) *Sph*I; (Q) *Xho*I; (R) *Aar*II; (S) *Aha*III; (T) *Nco*I.

4. Discussion

Bacteriocins from lactic acid bacteria are of importance in bioconservation of various foods. Moreover, the use of more than one LAB bacteriocin as a combination of biopreservative may have major applications in improving food safety [1]. In the present study, the inhibitory effect of the cell-free filtrates of each of the 20 isolates was evaluated.

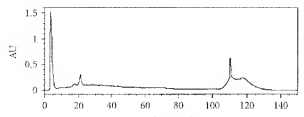

FIGURE 3: Elution pattern of chloroform extract from *S. thermophilus* T2 strain by reversed-phase high-performance liquid chromatography.

Antimicrobial activity was observed for 6 isolates, and only against Gram positive bacteria. The biochemical nature of the antibacterial molecule produced by *S. thermophilus* T2 was studied in both the cell-free supernatant and the chloroform extract. Our results showed that the molecule, produced by *S. thermophilus*, is peptidic since the antibacterial activity of the molecule was lost after digestion with proteolytic enzymes. However, the neutralization (pH 7) and addition of catalase or α-amylase to the cell-free supernatant did not result in the loss of the antilisterial activity. Our results showed also that the bacteriocin produced by *S. thermophilus* is heat stable (up to 30 minutes at 95°C); these results are similar to what has been reported for thoenicin [22]. In addition, the bacteriocin was stable over a wide pH range, this indicates that such bacteriocin may be useful in acidic as well as nonacidic food; similar pH stability results have been reported for propionicin PLG1 [14]. Growth and bacteriocin production profiles showed that the maximal bacteriocin production was measured by the end of the late-log phase. The level of production remained at a steady state during the stationary phase; similar results were obtained by Ivanova et al. [23]. However, bacteriocin production decreases after 24 hours of incubation after having reached maximum levels after 14 hours. This reduction could be a result of the inactivation of bacteriocin by extracellular proteases.

Preliminary characterization of the bacteriocin produced by *S. thermophilus* T2 was realized in the present study. It was found that the bacteriocin inhibits closely related Gram positive strains like *Listeria innocua* and *Enterococcus faecalis*. Activity against Gram negative was rarely reported for bacteriocin [24, 25]. Active substance from culture supernatant of *S. thermophilus* T2 was obtained according to the procedure described by Burianek and Yousef [16]. Chloroform was added to the cell-free supernatant in a separator funnel, the bacteriocin was concentrated at the interface between chloroform and the aqueous phase. This method effectively recovers higher bacteriocin yield and results in relatively clean preparations. Recovery of bacteriocin by the chloroform extraction was 10-fold higher when compared with ammonium sulphate precipitation (data not shown). The chloroform extraction procedure saves time , and it is easy to perform. This study allowed to underline the presence of at least one plasmid, of 4.2 kb as reported for many strains of *S. thermophilus* [21].

In conclusion, the study of autochthonous LAB will help to select the best candidates for improving the microbiological safety of traditional food products such as Raïb and may increase their shelf life. Such a collection could be used for construction of specific starter cultures for fermented food products.

Acknowledgment

This work was supported by a Ph.D. fellowship, within the PNE (Plan National Exceptionnel) Program administered by the Conference Régionale, Ministère de l'Enseignement Supérieur et de la Recherche Scientifique (Algeria). The authors sincerely thank Professor D. Guillochon, ProBioGEM laboratory.

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Full Length Research Paper

Antimicrobial activity of autochthonous lactic acid bacteria isolated from Algerian traditional fermented milk "Raïb"

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Twenty samples of traditional fermented milk "Raïb" were collected in eastern Algeria from individual household. They were evaluated for the presence of autochthonous bacteriocin-producing lactic acid bacteria. From 13 of these samples 52 strains of lactic acid bacteria were isolated, and shown to exhibit inhibitory activity against the indicator strain *Listeria monocytogenes*. Five of these inhibitor-producing isolates were selected for further study on the basis of their relatively wide antimicrobial spectrum. The inhibitory spectra of activity of the selected strains were evaluated against a range of Gram-positive and Gram-negative test organisms. *Listeria monocytogenes and Staphylococcus aureus* were the most sensitive indicator tested. All the antimicrobial compounds produced by the selected lactic acid bacteria were fully or partially inactivated by some of the proteolytic enzymes, but were unaffected by catalase which indicates their proteinaceous nature. The compounds were heat stable up to 120°C for 20 min, and were active from pH 3.0 to 10.0. Highest bacteriocin activity was recorded under acidic conditions and activity decreased with increasing alkalinity.

Key words: Traditional fermented milk, Raïb, lactic acid bacteria, bacteriocin.

INTRODUCTION

Fermented milk is a dairy product obtained by the fermentation of milk, which may have been made from products obtained from milk with or without any modification of their composition, via the action of appropriate microorganisms and which result in a lowering of the pH with or without coagulation. Production of traditional cheeses (el-Klila, Jben) and other fermented milk products such as raib (fermented milk), Iben (skimmed fermented milk), has a very long tradition in Algeria. Raib is made from the raw cow or goat milk. Milk fermentation, like many traditional fermenting processes, is spontaneous and uncontrolled and could be a valuable source of autochthonous Lactic Acid Bacteria (LAB) (Hamama, 1992; El Soda et al., 2003). The microbiological characteristics of several fermented milk have been studied in Indonesia (Hosono et al., 1989), South Africa (Beukes et al., 2001) and Morocco (Hamama. 1992).

Lactic acid bacteria play an important role in food fermentation processes. Raw foods such as milk, fruit, vegetables or meat are often preserved by lactic acid fermentation (Savadogo et al., 2006: Daeschel 1989). In such food products LAB have the capacity to perform fermentative activities, which may result in active inhibition of spoilage and pathogenic bacteria. The antimicrobial effect may be due to the production of a number of antimicrobial substances such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins (Hoover, 2000; Lindgren and Doborogosz, 1990). Bacteriocins are produced by some strains of LAB; they are antimicrobial peptides with activity against strains closely related to the producer micro-organism. Some bacteriocins are also active against Gram-positive food-borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus subtilis* and spores of *Clostridium perfringens*. For this reason, they have received much attention for use as natural or so-called 'biopreservatives' in foods in recent years (Savadogo et al., 2006: Savadogo et al., 2004). Bacteriocins of LAB have been classified into four

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structural classes, namely I, II, III and IV (Nes et al., 1996). Classes I and II are small, mainly hydrophobic and heat-stable peptides. Class I, the so-called lantibiotics, are post-translationally modified, while Class II, non-lantibiotic bacteriocins, are divided into three subcategories: Class IIa are the pediocin-like bacteriocins with strong antilisterial effects; Class IIb bacteriocins consist of two peptides, both required for full antimicrobial activity and Class IIc bacteriocins are secreted by a sec-dependent mechanism. Class III are high molecular weight, heat-labile protein bacteriocins. Class IV are complex bacteriocins, composed of a protein moiety plus one or more non-proteinaceous additions, e.g. lipid or carbohydrate groups required for activity (Nes et al., 1996).

The purposes of this study were to isolate bacteriocin-producing lactic acid bacteria from traditional fermented milk 'Raïb" samples and to determine their spectrum of activity against food-borne pathogens. The antimicrobial activity of previously identified bacteriocin-producing lactic acid bacteria against these pathogens was also determined. We report that several strains produce bacteriocins active against *L. monocytogenes*, *S. aureus*, and *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains and growth media

All strains used in this study were maintained as frozen stocks in 25% glycerol at -20°C and were propagated twice in broth for 16 h before experimental use. LAB isolates were selected and cultivated on de Man Rogosa Sharpe Agar (MRSA, Oxoid) at 30°C. Bacteria chosen as indicators were: *Staphylococcus aureus* ATCC 25293, *Listeria monocytogenes ATCC 7644*, *Bacillus cereus* ATCC 14578, *Bacillus subtilis* ATCC8, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25422 and *Pseudomonas aeruginosa* 27853 were propagated in Tryptic Soy Broth at 30°C.

Fermented milk sampling

Twenty samples of traditional fermented milk (Raïb) were collected from individual households of rural areas in eastern Algeria. Samples were collected in sterile small bottles and stored in laboratory under refrigeration at 5°C until they were used in experiments.

Selection procedure for LAB from fermented product

10 ml of each sample were aseptically added into 90 ml of sterile 0.9% NaCl solution and mixed thoroughly. Serial dilutions (10^{-1} to 10^{-8}) were performed and 1 ml aliquots of the appropriate dilutions were directly inoculated in triplicate on media for lactic acid bacteria, M17 (Terzaghi and Sandine, 1975) and MRS (de Man et al., 1960) adjusted to pH 5.5. After incubation at 30°C for 24 h and 3 days, representative strains of lactic acid bacteria were obtained from M17 and MRS plates of highest sample dilutions. Colonies were either randomly picked up or when the plate contained less than 10 colonies.

Detection of antagonistic activity

Isolated colonies of the assumed LAB isolates were screened for antimicrobial-producing activity essentially using the spot method as described by Spelhaug and Harlander (1989). An overnight culture of the test organism grown in MRS broth supplemented with 2.5% yeast extract (MRSY) was diluted 10-fold in 10 mmol l^{-1} Tris HCl (pH 7.0), and 2 ml aliquots were spotted onto MRS agar. Plates were incubated for approximately 24 h, until growth was evident, and then overlaid with 5 ml Trypticase soft agar (0.7% agar) seeded with 0.1 ml of an overnight culture of *L. monocytogenes* ATCC7644. Plates were incubated for an additional 18 h, and then checked for clear zones around spots of the putative producers.

Presumptive identification of bacteriocin-producing strains

Bacteriocin producing strains were Gram stained and examined microscopically for cellular morphology and Gram-stain phenotype. Catalase activity was tested by spotting colonies with 3% hydrogen peroxide. Growth was assayed in MRS broth at 10, 15, 37 and 45°C. Salt tolerance was tested with 6.5, 7.0 and 10% (w/v) NaCl in MRS broth. Growth of the strains was also studied, at pH 4.4 ant 9.6 in MRS broth (Schleifer and Kilpper-Balz, 1984). The configuration of lactic acid, hydrolysis of arginine and production of CO_2 from glucose were determined according to the methods described by Schillinger and Lucke (1987). Sugar fermentation reactions were performed using API 50CH test strips and 50CHL medium, according to the manufacturer's instructions (BioMe´rieux).

Sensitivity to heat, pH, and hydrolytic enzymes

Cell-free supernatants (CFS) from the lactic acid cultures were collected by centrifugation (7500 g, 10 min, 4°C) of overnight MRS broth cultures. The supernatant fluids were adjusted to pH 6.5 and exposed to heat treatments of 65°C for 40 min, 95°C for 20 min, and 120°C for 20 min, and then were tested for remaining antimicrobial activity. In order to determine the effect of pH on Semi-purified preparations of the bacteriocin were adjusted to various pH values in the range of 3 to 10. The pH-adjusted bacteriocin samples were incubated at 37°C for 20 min and then neutralized to pH 6 and tested for bacteriocin activity.

The following enzymes were tested for their hydrolytic activity on the antimicrobial compounds contained in the supernatants: proteinase K (2.6 U mg^{-1}), pronase E (22 U mg^{-1}), pepsin (16U mg^{-1}), catalase (adjusted to a final activity of 2600 U mg^{-1}), lipase (50 U mg^{-1}), and α-amylase (15 U mg^{-1}). The assays were performed at a final concentration of 0.5 mg ml^{-1} and at pH 6.5, except for pepsin (pH 3.0). Samples with and without enzymes were held at 35°C for 6 h and the remaining activity was determined by well-diffusion assay as described before using *L. monocytogenes* ATCC7644 as indicator strain.

Bacteriocin spectrum of inhibitory activity

The spectrum of activity against different bacteria (Table 2) was determined by the well-diffusion assay (schillinger and Lucke, 1989) and disk diffusion assay (Tagg and McGiven, 1971). The well-diffusion was conducted in TSA agar media overlaid with 7 ml of soft agar media which contained 4% inoculum of an overnight culture of the indicator strain wells, 4 mm in diameter, were cut into these agar plates and 300 µl of the culture supernatant of the potential producer strain were placed into each well (Figure 1). The plates were incubated for 24 h at 37°C and subsequently examined for zones of inhibition (Barefoot and Klaenhammer, 1983).

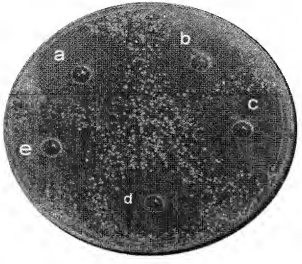

Figure 1. Inhibition of *Listeria monocytogenes* ATCC 7644 by the cell-free supernatants of the five producing isolates using the agar well-diffusion assay: *Lactobacillus plantarum* LB44a (well a), *Ln. mesenteroides* subsp. *mesenteroides* LM25a (well b), *Lactobacillus brevis* LM93b (well c), *Lactococcus lactis* subsp. *lactis* MB31a (well d) and *Lactobacillus asidophillus* LM65c (well e).

RESULTS

Detection of antimicrobial producing LAB

Putative antimicrobial-producing LAB isolated from fermented milk were detected using the spot method assay on the basis of their ability to inhibit growth of the indicator strain *L. monocytogenese* ATCC7644. According to the results, of a total of 20 different traditional fermented milk samples analysed, 13 samples presented strains of lactic acid bacteria that were found to produce bacteriocin-like substances. From each of these samples, 52 inhibitor-producing bacteria, which were presumed to be LAB, were isolated. 5 of these inhibitor-producing isolates were selected for further study on the basis of their relatively wide antimicrobial spectrum, and consistently released their activity into the CFS (Figure 1).

The five presumptive bacteriocin producers were characterised and identified to species level utilising carbohydrate fermentation profiles, biochemical and physiological characteristics (Table 1) details, these bacteriocin-producing strains were Gram-positive, catalase-negative two cocci and three rod. All strains were capable of growing at 15°C but not at 45°C, nor at pH 9·6 and nor in the presence of 10% NaCl (Table 1). Based on these characteristics, as well as on carbohydrate fermentation patterns (Table 1), the strains were presumptively identified as *Lactobacillus plantarum* (LB44a), *Lb. acidophilus* (LB65c), *Lb. brevis* (LB93b), *Leuconostoc* (LM25a) and *Lc. Lactis* (MB31a).

Sensitivity to proteolytic and lipolytic enzymes

The sensitivity of the antibacterial substances produced by lactic acid bacteria to α-chymotrypsin, trypsin, pronase E, proteinase K, pepsin, catalase, and lipase was determined in controlled and reproducible conditions shown in Table 3. All the compounds were fully or partially inactivated by some of the proteolytic enzymes, which indicate their proteinaceous nature.

In general, the inhibitory compounds produced by these strains presented different patterns of sensitivity. All of them were completely inactivated by α-chymotrypsin, pronase E, and pronase K. Only one was resistant to pepsin (strain LB44a), while the compounds produced by strains MB31a and LB93b, were partially inactivated after treatment with lipase, indicating that these inhibitory substances may have a lipid moiety in their chemical composition.

Inhibitory spectrum

The sensitivity of various Gram-positive and Gram negative bacteria to the CFS of the five producing isolates was determined using the well-diffusion assay (Table 2). The inhibitory spectrum of the CFS obtained from the five isolated bacteriocin-producing LAB tested against these bacteria included most notably *B. cereus* and *B. subtilis*, which were consistently inhibited by isolates LB44a and LB65c, although not to the same extent as some of the other bacteria tested. Whereas the CFS from the strain LB44a shown to inhibit Gram negative bacteria tested; *Escherichia coli* ATCC 25422 and *Pseudomonas aeruginosa* ATCC 27853.

CFS from bacteriocin-producer LB65c and LB44a was shown to have the broadest inhibitory spectrum of the producer stralns against these bacteria, while the CFS of producers, LM25a, LB93b and MB31a exhibited a narrower spectrum.

Temperature and pH stability of bacteriocins

The stability of the secreted inhibitory compounds was tested using different temperature treatments (Table 3). The inhibitory activity was shown to be completely unaffected following heat treatments at 65 and 95°C. The inhibitory compounds produced by isolates LB65c and LB93b were seen to be the most stable to heat treatments up to and beyond 100°C. LB93b maintains its activity even after treatment at 120°C for 20 min, a property which is typical for bacteriocins. The observed protease sensitivity and stability at high temperatures therefore conclusively identifies these compounds as bacteriocins.

The stability of the inhibitory activity was tested at different pH values (Table 3). The bacteriocins produced by isolates, L65c and LB44a showed greater pH tolerance

Table 1. Phenotypic characteristics of the bacteriocin-producing strains isolated from traditional fermented milk.

Characteristic	Strain designation				
	LB44a	LM25a	LB65c	LB93b	MB31a
Gram stain	+	+	+	+	+
Morphology	R	C	R	R	C
Catalase test	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+
Formation of: H2S	-	-	-	-	-
NH3 from arginine	+	-	-	+	+
Growth at:					
10°C	n	+	+	+	-
15°C	+	+	+	+	+
45°C	-	-	-	-	-
pH 4.4	-	+	+	+	+
pH 9.6	-	-	-	-	-
Growth in:					
6.5% NaCl	+	+	+	+	n
7.0% NaCl	-	+	-	-	-
10.0% NaCl	-	-	-	-	-
Gas from glucose	-	+	-	+	-
DL-Lactic acid	n	n	D	D	n
Carbohydrates					
Arabinose	-	-	-	+	-
Cellobiose	+	+	+	-	+
Esculin	+	-	+	+	n
Galactose	+	-	+	+	+
Gluconate	+	-	-	+	+
Glycerol	-	-	-	-	-
Inulin	+	-	-	+	n
Lactose	+	-	+	+	+
Maltose	+	+	-	-	+
Mannitol	+	-	+	-	+
Melezitose	+*	-	+	-	-
Melibiose	+	-	+	+	-
Raffinose	+	-	-	+	-
Rhamnose	+*	-	-	-	-
Ribose	+	+	-	+	+
Salicin	+	-	-		-
Sorbitol	+	-	-	-	-
Sucrose	+	+	+	-	+
Trehalose	+	+	-	-	+
Xylose	-	-	-	-	+
Identified as	*Lactobacillus plantarum*	*Ln. mesenteroides* subsp. *mesenteroides*	*Lb. acidophilus*	*Lb. brevis*	*Lc. lactis*

+ = Growth (+), - = no growth, +* = delayed fermentation.
R, Rod; C, Cocci; n, not performed.

and stability than those secreted by isolates (LM31a), (LB93b), and (LM25a).

DISCUSSION

Isolation and screening of microorganisms from naturally occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes. This is certainly true for lactic acid bacteria (LAB), which play an important role in a large number of various traditional food fermentations (El Soda et al., 2003; Vijai et al., 2004). Among these traditional

Table 2. Inhibitory spectrum of the pH neutralized cell-free supernatants of the LAB strains isolated from fermented milk, as determined with the well-diffusion assay.

Indicator strains	Inhibitory activity of producer strains				
	LB44a	LM25a	LB65c	LB93b	MB31a
Gram positive					
Bacillus .cereus ATCC 14578	++	-	+	-	-
Bacillus subtilis ATCC8	+	-	+	-	-
Staphylococcus aureus ATCC 25293	++	++	+	+	+++
Listeria monocytogenes ATCC 7644	+++	+++	+++	+++	+++
Ent. faecalis ATCC 19433	+	+	++	+	+
Gram nigaitve					
Escherichia coli ATCC 25422	++	-	++	-	-
Pseudomonas aeruginosa ATCC 27853	+	-	-	-	-

- = no inhibition zone, + = inhibition zone up to 4 mm, ++ = inhibition zone up to 10 mm: +++ = inhibition zone over 10 mm.

Table 3. Effect of heat treatment, pH and proteolytic enzymes on the antimicrobial compounds produced in the supernatant by selected lactic acid bacteria isolated from Algerian fermented milk[ab].

Treatment	% Activity[c]				
	LB44a	LM25a	LB65c	LB93b	MB31a
Heat Treatment					
65°C/40 min	100	100	100	100	100
95°C/20 min	100	100	100	100	100
100°C/20 min	50	50	100	100	50
120°C/20 min	50	50	50	100	50
pH					
3	50	50	50	00	50
4	50	50	100	50	50
5	100	50	100	50	100
6	100	100	100	100	100
7	100	100	100	100	100
8	100	50	100	00	50
9	50	50	50	00	00
10	50	00	50	00	00
Enzymes					
Pronase E	0	0	0	0	0
Proteinase K	0	0	0	0	0
α-Chymotrypsin	0	0	0	0	0
Pepsin	100	0	0	0	0
Lipase	100	100	100	50	50
Catalase	100	100	100	100	100

[a]All assays were conducted with *Listeria monocytogenes* ATCC 7644 as indicator strain.
[b]Producer strains are termed by the numbers of collection: *Lactococcus lactis subsp lactis* (MB31a), *Ln. mesenteroides* subsp *mesenteroides* (LM25a), *Lactobacillus brevis* (LM93b), Lactobacillus plantarum (LB44a) and *Lactobacillus asidophillus* (LM65c).
[c]Antimicrobial activity was expressed as the % of residual activity.

processes, fermented milk is known to be essentially fermented by LAB, although often a functional secondary flora develops. Some properties of LAB such as flavour and texture formation as well as inhibit pathogenic and spoilage microorganisms are especially important to the food and feed industries because of their applicability for a large variety of products. In addition, a large number of bacteriocins from lactic acid bacteria have been des-

cribed recently. While bacteriocin production has been reported from bacteria in milk products, fermented foods (Onda et al., 2003) and silage (Gollop et al., 2005)

In the present study, 52 lactic acid bacteria isolated from traditional fermented milk and were screened for bacteriocin production, from which, five bacteriocinogenic strains were identified and selected for further study, representing three isolates of *Lactobacillus brevis*, *Lactobacillus asidophillus*, *Lactobacillus plantarum* one *Leuconostoc* and one *L. lactis* isolates. This would indicate that a wide variety of bacteriocin-producing LAB are present on fermented milk, which therefore represent an abundant resource of such potentially useful bacteria.

The results of the Table 3 show that antibacterial compounds produced are inactive by all the proteolytic enzymes (pepsin, trypsin, α-chymotrypsin), indicating that the inhibitory compounds are proteinaceous nature, a general characteristic of bacteriocin. No zone of inhibition was discovered after stake in the presence of our extracts with these various enzymes. It has been reported that other bacteriocins than nisine are generally inactivated by an array of proteolytic enzymes including those of pancreatic origin (trypsin and α-chymotrypsin) and sometimes of gastric origin (pepsin). This high sensitivity of lactic acid bacterial bacteriocins to metabolic proteolytic enzymes is very interesting with respect to food safety, since it means that the ingestion of bacteriocins will not alter digestive tract ecology and also will not cause risks related to the use of common antibiotics (Bromberg et al., 2004).

It is interesting to note that the compound produced by the strains LB44a and MB31c, were partially inactivated after treatment with lipase, indicating that these inhibitory substances may have a lipid moiety in their chemical composition. Some bacteriocins produced by bacteria of the genus *Lactobacillus* are sensitive to non-proteolytic enzymes. Plantaricin B is inactivated by a lipase and by an α-amylase, and plantaricin S is inactivated by glycolytic, lipolytic and phospholipolytic enzymes (Jéminez-Diaz et al., 1993). These observations indicate that the active part of bacteriocins of lactobacilli may be chemically heterogeneous, which could signify that the term bacteriocin covers a set of chemically varied substances.

The inhibitory compounds produced by the five isolates demonstrated a high resilience to heat treatments ranging in temperature from 30 to 120°C (Table 2). In the other hand the bacteriocins were shown to be stable over a broad pH range with all peptides maintaining some antimicrobial activity within the pH range of pH 3 to 10. According to Tagg et al. (1976) bacteriocins differ greatly with respect to sensitivity to pH. Many of them are considerably more tolerant of acid than alkaline pH values. In the present study bacteriocin produced by the strain LB65c exhibited the same profile and was active at pH values between 4 - 9. Maximum inhibitory activity was demonstrated at pH 4 and 5. Similar properties have been reported for other bacteriocins including lactacin, lactacin 27, acidolin, pediocin A, and pediocin PA-1 (Hastings et al., 1996). These bacteriocins were also stable over a wide range of pH. Piard and Desmazeaud (1992) also reported that temperature stability is very convenient if the bacteriocin is to be used in food preservative, because many processing procedures involve a heating step, and cold is one of the most popular preservation procedures. Furthermore, activity at neutral pH constitutes an advantage over other bacteriocins used as food preservatives and particularly over nisine, whose maximal solubility and stability are at pH 2, with these parameters decreasing significantly as the pH increases.

Generally, the bacteriocins from LAB were shown to be ineffective against Gram negative bacteria. The partially purified bacteriocin preparations from the strain (LB44a) showed broad antimicrobial activity including against Gram-negative *Pseudomonas* and *E. coli* strains (Table 2). Earlier, Sumar et al. (1998) also reported the inhibitory action of bacteriocin of *L. plantarum* against Gram-negative strains. Lactobacillus bacteriocins are found within each of the four major classes of antimicrobial proteins produced by LAB and the lactobacilli produce many different bacteriocins activity (Alpay Karaoglu et al., 2003). Among the lactobacilli, there has been great interest in *L. plantarum*, due to the potential application of the microorganism as a starter bacterium for a variety of fermented foods (McKay and Baldwin, 1990). The bacteriocins produced from *L. plantarum* have been found to be inhibitory towards closely related LAB, particularly the mesophilic and thermophilic lactobacilli (Sumar et al., 1998)

Of all the indicator strains tested, *L. monocytogenes and S. aureus*, were the most sensitive, being inhibited by all five strains. However the cultures that produced "high" inhibition zones against *L. monocytogenes* were: LB44a, LB65c and LB93b. Therefore, the high sensitivity of the *Listeria* strain to the bacteriocins produced by our isolates is not surprising, since Daeschel et al. (1989) screened many bacteriocin-producing lactic acid bacteria for inhibition of *Listeria* species and found that some of them were able to produce an antimicrobial substance that was active against *Listeria monocytogenes*.

Conclusion

The analysis of antimicrobial activity of LAB isolated from a collection of LAB was made by isolating them from traditional fermented milk that is manufactured according to the local tradition without using any known starter culture. Analysis of LAB from the collection of natural isolates revealed that they produce bacteriocins.

The antimicrobial activity of the bacteriocins produced by the lactic acid bacteria isolated in this research could act as a barrier to inhibit food spoilage and/or growth of pathogenic microorganisms in foods. Considerable effort

has recently been focussed on the understanding of the structure, the genetic organization and the mode of action of several bacteriocins. There has been a concomitant development in the description of new bacteriocins, whose biochemical and genetic characterization should lead to the discovery of important elements for the elucidation of structure/function relationships in these substances.

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preliminary communication

Production, Purification, Stability and Efficacy of Bacteriocin from Isolates of Natural Lactic Acid Fermentation of Vegetables

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Summary

The antimicrobial activity of partially purified bacteriocin produced during natural lactic acid fermentation of carrot, radish and cucumber was assessed and characterized. Out of ten strains, the isolated strain CA 44 of *Lactobacillus* genus from carrot fermentation produced bacteriocin with maximum antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*, though it was more effective against *E. coli* than others. Bacteriocin was stable at up to 100 °C but its activity declined compared to that at 68 °C and was completely lost at 121 °C. The maximum antimicrobial activity was retained within the pH range of 4–5, but it was adversely affected by the addition of papain. Bacteriocin was also effective against *B. cereus* in different fruit products (pulp, juice and wine) indicating its potential application as a biopreservative in fruit products.

Key words: antimicrobial, bacteriocin, lactic acid fermentation, *Lactobacillus*, *Staphylococcus*, *Bacillus cereus*, *E. coli*, pathogenic microorganism, stability, biopreservative

Introduction

Preservation of vegetables by lactic acid fermentation is an ancient practice involving lactic acid bacteria (LAB), which predominantly produce lactic acid besides certain compounds such as bacteriocin, which has antimicrobial activity against other groups of microorganisms. The antimicrobial activity of bacteriocins produced by LAB has been detected in foods such as dairy products, meats, barley, sourdough, red wine, fermented vegetables, *etc.* (*1–5*). Therefore, the strains of lactic acid bacteria have also potential to act as a biopreservative or natural food preservative (*6–8*). The bacteriocins produced inhibited food spoilage and pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *B. subtilis*, *Listeria monocytogenes* and *Clostridium perfringens* which are recalcitrant to traditional food preservation method (*9*). The use of bacteriocins or the microorganisms that produce them is attractive to the food industry in the face of increasing consumer demand for natural products and the growing concern about foodborne diseases. It has also necessitated the need to exploit the biologically derived antimicrobial substances produced by LAB. It is not clear if any bacteriocin is produced in the vegetables fermented by LAB in natural or inoculated fermentation. The bacteriocin produced by the strains isolated from naturally fermented vegetables has neither been characterized nor checked for its efficacy in various food products. Therefore, keeping in view the above objectives the present investigations were carried out and the results obtained are discussed here.

Materials and Methods

Fermented vegetables

Vegetables (carrot, radish and cucumber) procured from the markets were washed, peeled and grated/sliced.

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The grated carrot and radish were fermented with dry salt 2 % (by mass) at 27 °C, whereas sliced cucumbers were fermented in 3 % (by mass per volume) brine at 32 °C. Predominant microflora were isolated from these samples.

Pathogenic bacterial cultures

Standard bacterial cultures, *viz. Escherichia coli* (0165), *Staphylococcus aureus* (B-43-5) and *Bacillus cereus* procured from Central Research Institute (CRI), Kasauli, were used in bacteriocin screening procedures and all the cultures were maintained as per the recommended practices.

Isolation and identification of bacteriocin producing bacteria

The bacteriocin producers from naturally fermented carrot, radish and cucumber were isolated by pour plate method technique as per the conventional method (*10*) using MRS agar. After incubation for 24–48 h at 32 °C, typical colonies were isolated and purified. The isolates were differentiated on the basis of their morphological, cultural and physiological characteristics such as oxidase test, utilization of citrate as a sole carbon source and catalase test (*10,11*), and accordingly were tentatively identified up to the genus level (*12*).

Screening of isolates for antimicrobial activity

Antimicrobial activity of the bacterial isolates against all the pathogenic microorganisms was determined by well diffusion method (*13–16*) under aerobic conditions. Agar plates were inoculated with 100 μL of each target microorganism after growing them in a broth and diluting appropriately. Wells (3 mm) were cut into the plates and 100 μL of cell-free culture supernatant fluid of the isolated strain was placed into each well. The inhibitory activity against *E. coli* was tested on EMB agar whereas *Staphylococcus aureus* and *Bacillus cereus* were tested on nutrient agar. Plates were kept at cool temperature for 2 h and then incubated at 37 °C for 24 h. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells. The bacterial isolate showing the widest zone of inhibition against the target microorganism was selected for further studies.

Partial purification of bacteriocin

Isolated strain having maximum antimicrobial zone was grown in MRS broth at 37 °C for 24 h. After incubation, the broth was centrifuged at 5000 × *g* for 10 min and the cells were separated out. Supernatant was used as a crude bacteriocin. Different concentrations of ammonium sulphate were added to the supernatant. After stirring on a magnetic stirrer, it was kept undisturbed at 4 °C overnight. Precipitates formed were collected by centrifugation at 10 000 × *g* for 10 min and redissolved in 20 mmol sodium phosphate buffer with pH=6.0. Inhibition zone of different fractions was recorded in comparison with the crude bacteriocin.

Characterization of bacteriocin

Heat stability

A volume of 5 mL of bacteriocin in different test tubes was overlaid with paraffin oil to prevent evaporation and then heated at 68 and 100 °C for 10 and 20 min, respectively, and at 121 °C for 15 min under pressure. The heat-treated bacteriocin samples were then assayed for antimicrobial activity as described earlier.

Effect of pH

A 5-mL aliquot of partially purified bacteriocin was taken in test tubes and the pH values of the contents were adjusted to 2–9 individually, using either diluted NaOH or HCl (1 M NaOH or 1 M HCl solution). After allowing the samples to stand at room temperature for 2 h the activity was assayed as described earlier.

Effect of proteolytic enzyme (papain)

A 5-mL aliquot of bacteriocin preparation was taken in test tubes and treated with papain (100 TU) 1 mg/mL at pH=7. The test tubes with and without the enzyme (control) were incubated for 2 h at 37 °C and heated for 3 min at 100 °C to denature the enzyme. Both the control and the samples were assayed for antimicrobial activity by using well diffusion method.

Determination of preservative effect of bacteriocin

The food products, *viz.* juice (apple), pulp (apricot) and prepasteurized wine (plum) were sterilized and inoculated with *Bacillus cereus* at 10^8 CFU/mL. Initial count of inoculated samples was recorded and bacteriocin supernatant at a concentration of 0.05 to 0.5 % was added. After 24 and 72 h, the plate count was recorded and compared with the control (without bacteriocin).

Results and Discussion

Based on morphological and biochemical tests, all the isolates were identified as belonging to lactic acid bacteria (LAB) group except RA33, which was identified as yeast. The isolate CA44 (giving maximum antimicrobial activity) was Gram-positive, rod shaped, negative for catalase and peroxidase test, having circular and white colonies on the MRS media. The strain was also positive for galactose, arabinose, mannitol, sorbitol, sucrose, glucose, trehalose, lactose, raffinose and negative for maltose, citrate and arginine test. Isolate CA44 from carrot produced the maximum inhibition zone against all the tested microorganisms and was maximum against *E. coli*. The best conditions for bacteriocin production by *Lactobacillus plantarum* in batch fermentation were the salt concentration ranging from 2.3 to 2.5 % and temperature ranging from 22–27 °C (*17*). *Lactobacillus plantarum* strain isolated from fermented carrots which produced bacteriocin with antibacterial activity against *Staphylococcus aureus* and spheroplasts of Gram-negative bacteria (*18*) and *Lactococcus lactis* ssp. *cremoris* was also isolated from radish fermentation (*1*).

An increase in antimicrobial activity after partial purification of crude bacteriocin by ammonium sulphate precipitation took place (Fig. 1). The fraction with the highest bacteriocin activity was precipitated with 20–30 %

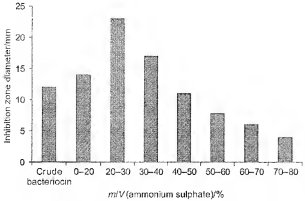

Fig. 1. Increase in antimicrobial activity of bacteriocin from *Lactobacillus* sp. isolate (CA44) using ammonium sulphate fractionation

(by mass per volume) ammonium sulphate. The antimicrobial activity (in terms of inhibition zone diameter) increased from 12 to 23 mm. There was 1.91-fold increase in the partially purified bacteriocin activity than that of crude bacteriocin. Earlier, the inhibitory activity of bacteriocin isolated from malted barley was precipitated from cell free supernatant using 40 % ammonium sulphate saturation, and resuspended in 2 mmol sodium phosphate buffer, pH=6.0 and purified using chromatography (*19*).

Partially purified bacteriocin was found to be stable at 68 °C for up to 20 min. At 100 °C for 10 min it could retain 55 % of antimicrobial activity, while at the same temperature for 20 min, only 28 % of activity could be retained (Table 1). However, after incubation for 15 min at 121 °C, the complete loss of activity took place. Compared to the earlier reports on bacteriocin, residual activity was lower in our study than reported earlier (*20*). Furthermore, since tolerance of bacteriocin to heat is known to depend on the stage of purification, pH, presence of culture medium, other protective components, *etc.* that might have influenced the antimicrobial activity in our findings too. The heat stability of bacteriocin discussed here indicates that it could be used as biopreservative in combination with thermal processing to preserve the food products. Furthermore, when comparatively low temperature is employed for processing compared to high temperature being used at present, the retention of nutrients would be higher. However, more studies on these aspects are needed.

The partially purified bacteriocin showed maximum activity against the target microorganisms at pH=5.0 (Fig. 2), but after pH=5.0 the activity of the bacteriocin gradually but continuously decreased. At pH=9.0, the antimicrobial activity was drastically reduced to more than 2.5 times that of the control. Thus, the bacteriocin was found active over a wide pH range with the highest activity at low pH range of 4–5. Earlier, the bacteriocin produced by a newly isolated *Bacillus* species strain 8A was found active over a pH range of 5–8 but was inactivated when incubated outside these limits (*9*). Another bacteriocin produced by *Lactococcus lactis* D53 and 23 was active over a wide pH range with the highest activity shown at low pH range of 3–5 (*13*), as was the case with the bacteriocin from *Pediococcus* sp. (*21*). Bacteriocin activity was completely lost when treated with proteolytic enzyme (papain), which is in agreement with the earlier report (*22*). The bacteriocin pediocin ACH from *Pediococcus acidilacti* was sensitive to proteolytic enzymes and was completely inactivated by several proteolytic enzymes (*22,23*). The stability of bacteriocin to different conditions reflects that such compounds can withstand the conditions normally encountered in food processing, so would remain effective during processing.

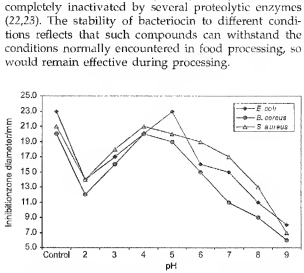

Fig. 2. Effect of pH on antimicrobial activity of partially purified bacteriocin from *Lactobacillus* sp. isolate (CA44)

The partially purified bacteriocin from isolate CA44 was also tested for preservative effect against *B. cereus* (Table 2), and clearly the preservative effect in juice, wine and pulp increased with the increase in the concentration of bacteriocin. Maximum reduction of *Bacillus cereus* population of 92 % was observed in wine followed by juice (87 %) and pulp (63 %) at a concentration of 0.5 %.

Table 1. Effect of temperature on antimicrobial activity of partially purified bacteriocin from isolated *Lactobacillus* sp. (CA44)

Temperature/°C	*t*/min	Inhibition zone diameter/mm		
		E. coli	*B. cereus*	*S. aureus*
68	10	23 (100)	19 (100)	20 (95)
	20	22 (95)	19 (100)	20 (95)
100	10	15 (65.21)	13 (68.42)	11 (55)
	20	10 (43.47)	9 (47.36)	6 (28.57)
121	15	0	0	0
Control (without heat treatment)	–	23	19	21

Values in parentheses represent retention of antimicrobial activity (in %)

Table 2. Preservative effect of partially purified bacteriocin from *Lactobacillus* sp. isolate (CA44) in juice, wine, and pulp against *Bacillus cereus*

ψ(bacteriocin)/%	Preservative effect*/%		
	Juice	Wine	Pulp
Control	0	0	0
0.05	12	16	10
0.1	34	37	17
0.2	50	55	29
0.3	69	72	57
0.4	83	86	60
0.5	87	92	63

*Reduction of population/% = $\frac{\text{Reduction in microbial count}}{\text{Total count in control}} \times 100$

However, in control (without bacteriocin), no reduction was observed in the count of *B. cereus*. The results (Fig. 3) further revealed that microbial count drastically decreased in wine and the same pattern was followed in juice too. In pulp, only a concentration of bacteriocin above 0.2 % drastically decreased the microbial count. Highest antimicrobial activity of bacteriocin against the target microorganism in wine could partly be attributed to inhibitory effect of ethanol. Briefly, the results indicate that bacteriocin possessed several desirable characteristics of a biopreservative.

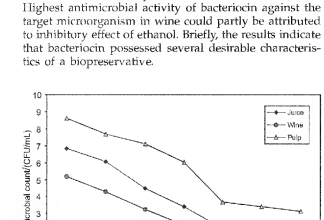

Fig. 3. Reduction in population of *Bacillus cereus* in juice, wine and pulp with the addition of bacteriocin

Conclusion

The study revealed that bacteriocin from *Lactobacillus* sp. isolated from natural lactic acid fermentation of vegetables possesses a wide spectrum of inhibitory activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. Therefore, it has a potential for application as a biopreservative in different food products as such or in combination with other preservation methods. Since lactic acid fermentation is employed mostly for development of products, especially for flavour and taste of the fermented products, the production of bacteriocin in such products assumes more significance as biopreservative apart from imparting probiotic effect to the product.

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